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May 24, 2001

BOX PCT

Assistant Commissioner
for Patents
Washington, D.C. 20231

PCT/AU99/01048-filed
November 24, 1999

Re: Application of **Mark Brenton NOTTLE, Ranald CAMERON, Luke Francis Sharkerley BEEBE, Alan Weaver BLACKSHAW** and **Hiroshi NAGASHIMA**
entitled "**CRYOPRESERVATION OF OOCYTES AND EMBRYOS AND METHOD FOR PRODUCING ANIMALS INVOLVING THE SAME**"
BRESAGEN LIMITED and THE UNIVERSITY OF QUEENSLAND
Our Ref: **Q-64691**

Dear Sir:

The following documents and fees are submitted herewith in connection with the above application for the purpose of entering the National stage under 35 U.S.C. § 371 and in accordance with Chapter II of the Patent Cooperation Treaty:

- ☐ an executed Declaration and Power of Attorney.
- ☐ an English translation of the International Application.
- ☐ sheets of drawings.
- ☐ an English translation of Article 19 claim amendments.
- ☑ an International Preliminary Examination Report (IPER).
- ☐ an executed Assignment and PTO 1595 form.
- ☐ a Form PTO-1449 listing the ISR references, and a complete copy of each reference.
- ☑ a Preliminary Amendment.

The Declaration and Power of Attorney, Assignment, Form PTO-1449 listing the International Search Report (ISR) reference

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and a complete copy of the reference will be submitted at a later date.

It is assumed that copies of the International Application and International Preliminary Examination Report (and any Articles 19 and 34 amendments) as required by § 371(c) will be supplied directly by the International Bureau. However, for the Examiner's convenience, a copy of each of these documents is provided herewith.

Priority is claimed from **November 24, 1998**, based on Australian Application No. **PP 7299**.

Applicants claim benefit of small entity status in accordance with 37 C.F.R. § 1.27.

The Government filing fee is calculated as follows:

Total claims	18	- 20 =		x	$9.00	=	$.00
Independent claims	1	- 3 =		x	$40.00	=	$.00
Base Fee							$500.00
TOTAL FEE							$500.00

A check for the statutory filing fee, in the amount of $500.00, is attached hereto.

The Assistant Commissioner is hereby directed and authorized to charge or credit any difference or overpayment to Deposit Account No. 19-4880. The Assistant Commissioner is also hereby authorized to charge any fees under 37 C.F.R. §§ 1.16, 1.17 and 1.492 which may be required during the entire pendency of the application to Deposit Account No. 19-4880. A duplicate copy of this transmittal letter is attached.

Respectfully submitted,

Gordon Kit
Registration No. 30,764

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Mark B. NOTTLE et al

	CHAPTER II of	
Appln. No.:	**PCT/AU99/01048**	Group Art Unit: **000**
Filed:	**May 24, 2001**	Examiner: **Unknown**

For: **CRYOPRESERVATION OF OOCYTES AND EMBRYOS AND METHODS FOR PRODUCING ANIMALS INVOLVING THE SAME**

PRELIMINARY AMENDMENT

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

Prior to examining the above-identified application, please amend the application as follows.

IN THE SPECIFICATION:

Please amend the specification as follows:

Page 1, before line 4, insert

-- This application is a 371 of PCT/AU99/01048 filed November 24, 1999. --.

IN THE CLAIMS:

Please cancel Claims 1-14.

Please add the following new claims:

-- Claim 15. A method for cryopreservation of oocytes or embryos, which comprises the steps of:

(A) centrifuging oocytes or embryos to polarize cytoplasmic lipid outside of cells constituting said oocytes or embryos;

(B) subjecting the resulting lipid polarized oocytes or embryos to low temperature conditions sufficient to freeze the oocytes or embryos prior to lipid depolarization, wherein said subjecting is carried out in the presence of a cryoprotectant agent; and

(C) storing the resulting frozen lipid polarized oocytes or embryos at a low temperature to obtain cryopreserved oocytes or embryos.

Claim 16. The method of Claim 15, wherein said embryos are zona intact embryos.

Claim 17. The method of Claim 15, wherein said oocytes or embryos are obtained from a companion animal or domestic/livestock animal.

Claim 18. The method of Claim 17, wherein said companion animal is a dog or cat.

Claim 19. The method of Claim 17, wherein said domestic/livestock animal is selected from the group consisting of a horse, cow, sheep, goat, llama and alpaca.

Claim 20. The method of Claim 17, wherein said oocytes or embryos are porcine oocytes or porcine embryos.

Claim 21. The method of Claim 15, wherein said embryos are vitrified in the presence of a solution comprising one or more cryoprotectant agent.

Claim 22. The method of Claim 21, wherein said cryoprotectant agent is selected from the group consisting of dimethylsulfoxide, propylene glycol, ethylene glycol, glycerol, PVP, sucrose, trehalose, Ficoll, acetamide and egg yolk.

Claim 23. A method for producing live animals from embryos which comprises the steps of:

(A) thawing a cryopreserved zona intact embryo obtained by the method of Claim 16; and

(B) introducing the resulting thawed embryo into the uterus or fallopian tube of a pregnancy competent female pig such that said female pig becomes pregnant with said embryo, wherein after a pregnancy term, the resulting pregnant female pig gives birth to a live piglet.

Claim 24. An animal produced from a cryopreserved oocyte or embryo obtained by the method of Claim 15.

Claim 25. A piglet obtained by the method of Claim 23.

Claim 26. The animal of Claim 24, wherein said animal is a pig.

Claim 27. A cryopreserved oocyte or embryo obtained by the method of Claim 15.

Claim 28. The method of Claim 21, wherein said embryos are vitrified by freezing in liquid nitrogen.

Claim 29. The method of Claim 28, wherein said oocytes or embryos are frozen in a freezing vessel.

Claim 30. The method of Claim 29, wherein said freezing vessel is a cryologic vial or freezing straw.

Claim 31. The method of Claim 30, wherein said freezing straw is an open pulled straw in which the oocytes or embryos are located by capillary action.

Claim 32. The method of Claim 16, wherein said embryos are in the morulae to mid-blastocyst stage. --

PRELIMINARY AMENDMENT
CHAPTER II of PCT/AU99/01048

IN THE ABSTRACT:

Please insert the Abstract attached hereto.

REMARKS

The specification has been amended to insert formal matter, Claim 1-14 has been cancelled and presented as new Claims 15-32 for clarification purposes only, and the Abstract has been inserted in order to make the application consistent with U.S. patent practice. Hence, the amendment of the specification, the cancellation of Claims 1-14, the addition of new Claims 15-32 and the Abstract do not constitute new matter.

In view of the amendments to the specification, the cancellation of Claims 1-14, the addition of new Claims 15-23 therefor and the insertion of the Abstract, allowance is respectfully requested.

The Examiner is invited to contact the undersigned at his Washington telephone number on any questions which might arise.

Respectfully submitted,

Gordon Kit
Registration No. 30,764

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Date: May 24, 2001

A P P E N D I X

Marked-up Version of Amended Specification

IN THE CLAIMS:

Claims 1-14 are cancelled.

New Claims 15-32 are added as follows:

-- Claim 15. A method for cryopreservation of oocytes or embryos, which comprises the steps of:

(A) centrifuging oocytes or embryos to polarize cytoplasmic lipid outside of cells constituting said oocytes or embryos;

(B) subjecting the resulting lipid polarized oocytes or embryos to low temperature conditions sufficient to freeze the oocytes or embryos prior to lipid depolarization, wherein said subjecting is carried out in the presence of a cryoprotectant agent; and

(C) storing the resulting frozen lipid polarized oocytes or embryos at a low temperature to obtain cryopreserved oocytes or embryos.

Claim 16. The method of Claim 15, wherein said embryos are zona intact embryos.

Claim 17. The method of Claim 15, wherein said oocytes or embryos are obtained from a companion animal or domestic/livestock animal.

Claim 18. The method of Claim 17, wherein said companion animal is a dog or cat.

Claim 19. The method of Claim 17, wherein said domestic/livestock animal is selected from the group consisting of a horse, cow, sheep, goat, llama and alpaca.

Claim 20. The method of Claim 17, wherein said oocytes or embryos are porcine oocytes or porcine embryos.

Claim 21. The method of Claim 15, wherein said embryos are vitrified in the presence of a solution comprising one or more cryoprotectant agent.

Claim 22. The method of Claim 21, wherein said cryoprotectant agent is selected from the group consisting of dimethylsulfoxide, propylene glycol, ethylene glycol, glycerol, PVP, sucrose, trehalose, Ficoll, acetamide and egg yolk.

Claim 23. A method for producing live animals from embryos which comprises the steps of:

(A) thawing a cryopreserved zona intact embryo obtained by the method of Claim 16; and

(B) introducing the resulting thawed embryo into the uterus or fallopian tube of a pregnancy competent female pig such that said female pig becomes pregnant with said embryo, wherein after a pregnancy term, the resulting pregnant female pig gives birth to a live piglet.

Claim 24. An animal produced from a cryopreserved oocyte or embryo obtained by the method of Claim 15.

Claim 25. A piglet obtained by the method of Claim 23.

Claim 26. The animal of Claim 24, wherein said animal is a pig.

Claim 27. A cryopreserved oocyte or embryo obtained by the method of Claim 15.

Claim 28. The method of Claim 21, wherein said embryos are vitrified by freezing in liquid nitrogen.

Claim 29. The method of Claim 28, wherein said oocytes or embryos are frozen in a freezing vessel.

Claim 30. The method of Claim 29, wherein said freezing vessel is a cryologic vial or freezing straw.

Claim 31. The method of Claim 30, wherein said freezing straw is an open pulled straw in which the oocytes or embryos are located by capillary action.

Claim 32. The method of Claim 16, wherein said embryos are in the morulae to mid-blastocyst stage. --

ABSTRACT

A method for the cryopreservation of oocytes or embryos, which comprises centrifugation of oocytes or embryos to polarize cytoplasmic lipid outside the oocyte or embryonic cells, subjecting the oocytes or embryos to low temperature conditions in the presence of a cryoprotectant which results in freezing of the oocytes or embryos prior to lipid depolarization, followed by low temperature storage of the frozen lipid polarized oocytes or embryos are described, as are oocytes and embryos produced according to such methods, and methods for producing live animals.

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PCT/AU99/01048
JC18 Rec'd PCT/PTO 24 MAY 2001

CRYOPRESERVATION OF OOCYTES AND EMBRYOS AND METHODS FOR PRODUCING ANIMALS INVOLVING THE SAME

The present invention relates to methods for the cryopreservation of oocytes and embryos, use of oocytes and embryos, and methods for producing live animals from such embryos.

The production of live animals from frozen thawed embryos has been described for a number of species including cattle and sheep.

However the production of live animals such as piglets from frozen/thawed oocytes and embryos remains problematic. In relation to pigs for example, a small number of piglets have been produced using conventional techniques and all have been from perihatching embryos, and not zona intact porcine embryos. The perihatching embryo stage is unsuitable for most uses because the embryos are not surrounded by an intact zona pellucida and are subject to bacterial and viral infection. This is an all important requirement for the import/export of genetic material. Such protocols require embryos to be surrounded by an intact zona pellucida because it protects against bacterial and viral infection as mentioned above, thus reducing the risk of disease transmission.

In other animals such as companion animals (for example dogs and cats), and domestic/livestock animals (for example horses, goats, llamas and alpacas), the production of live animals from frozen/thawed oocytes and embryos has been very problematic with a low success rate. This is largely because early stage embryos for most species contain significant levels of lipid.

The successful cryopreservation of animal oocytes and embryos, remains largely illusionary. In pigs in particular, cryopreservation techniques used for cryopreservation of embryos from other species are generally not successful.

In one prior proposal, animal embryos were subject to conditions which reduce the level of lipid in the embryo. In this proposal lipid was forced from the embryonic cells, resulting in a layer of lipid between the blastomeres and the zona pellucida. This lipid was moved by

aspirating a lipid from the embryo using micro manipulation techniques. Removing lipids from embryos requires considerable technical skill, as well as much complexity.

The present invention seeks to overcome the problems associated with cryopreservation of oocytes and embryos, and seeks to provide simple, convenient and easily performed methods for the cryopreservation of oocytes and embryos, such as zona intact porcine embryos, and for producing live animals therefrom.

Summary of Invention

In accordance with a first aspect of this invention there is provided a method for the cryopreservation of oocytes or embryos, which comprises centrifugation of oocytes or embryos to polarise cytoplasmic lipid outside the oocyte or embryonic cells, subjecting the oocytes or embryos to low temperature conditions in the presence of a cryoprotectant which results in freezing of the oocytes or embryos prior to lipid depolarisation, followed by low temperature storage of the frozen lipid polarised oocytes or embryos.

Preferably, the oocytes or embryos are vitrified by freezing in liquid nitrogen or other very cold fluid or gas which allows rapid temperature reduction.

In accordance with another aspect of this invention there is provided a method for producing animals from embryos which comprises thawing a cryopreserved lipid polarised embryo and thereafter transferring the embryo to a synchronised recipient female, and allowing the embryo to develop to term to give rise to live animals.

Detailed Description of the Invention

The present invention provides for the cryopreservation of animal embryos, for example zona intact porcine embryos, which hitherto have not been amenable to cryopreservation, and more particularly the successful production of animals from the cryopreseved embryos.

Cryopreserved oocytes can, on thawing, be fertilised, or genetically manipulated and fertilised, and then transferred into a pregnancy competent female to give live animals.

The inventors have surprisingly found that centrifugation of oocytes and embryos, such as zona intact porcine embryos, which polarises cytoplasmic lipid outside the oocyte and embryonic cells, followed by exposure to low temperature conditions, preferably vitrification, in the presence of cryoprotectant, enables successful cryopreservation of the polarised oocytes and embryos which maintains their viability such that on implantation into the uterus of a pregnancy competent female animals, progeny animals can develop. Accordingly, in a first aspect of this invention there is provided a method for the cryopreservation of animal oocytes embryonic cells comprising centrifugation of oocytes and embryos to polarise cytoplasmic lipid outside the oocyte or embryonic cells, subjecting the oocytes or embryos to low temperature conditions in the presence of a cryoprotectant which results in freezing of the embryo prior to lipid depolarisation, followed by low temperature storage of the frozen lipid polarised oocyte or embryo cells.

Embryos which may be subject to the methods of the present invention include zona intact embryos (blastomeres) from the oocyte stage, through to late blastocysts, including morulae to mid-blastocysts stage, and hatched (non-zona intact) blastocysts.

Oocytes and embryos may be from any animal, that is any mammal, including companion animals (for example dogs and cats), domestic/livestock animals (for example horses, cows, sheep, goats, pigs, llamas, and alpacas), laboratory animals (for example mice, rats, and monkeys), and humans. In a preferred aspect the invention relates to pigs, that is pig oocytes and embryos.

Oocytes and embryos may be recovered from donor animals by surgical or non-surgical methods. Non surgical methods can be used to recover oocytes and embryos from live cattle, but surgical methods are used for recovery from some other live animals, including pigs. For example, embryos may be surgically recovered from pigs within one to six days following mating. Alternatively, for livestock animals, oocytes and embryos may be flushed from reproductive tracts of slaughtered female animals. A second alternative is to obtain immature oocytes from the ovaries of slaughtered animals, and mature and fertilise them in vitro. The embryos obtained by any of these procedures may be briefly cultured in a medium standardly used for oocyte and/or embryo culture to an appropriate stage. Whilst in

no way essential, it is generally desirable to briefly culture embryos prior to the methods of this invention.

The oocytes and embryos are cultured in a cryoprotectant-containing solution prior to vitrification. The oocyte and embryos need only be incubated in the cryoprotectant solution for a short period of time, for example from two minutes to one hour, more preferably from two minutes to 30 minutes, still more preferably from 3 minutes to 20 minutes.

Oocytes and embryos may be incubated in a cryoprotectant-containing solution either prior to, during or after centrifugation, or both.

The cryoprotectant-containing solution in which oocytes and embryos are incubated either prior to, during centrifugation, or after centrifugation, may contain any standard cryoprotectant established for use in the freezing of animal oocytes and embryos, including glycerol, ethylene glycol, dimethylsulfoxide, propylene glycol and polyvinyl pyrrolidine, sucrose, trehalose, Ficoll, acetamide, egg yolk and the like. The concentration of cryoprotectant is used in an amount sufficient to replace to at least some extent water within the oocyte or embryo, such that on rapid freezing ice crystal formation is prevented. By way of example, cryopreservatives may be present in an amount from 0.5M to 8M. One or more cryoprotectants may be used. The time in which oocytes and embryos may be incubated in a cryoprotectant solution following centrifugation is insufficient to allow lipid repolarisation into the tissues of the oocyte or embryo.

Oocytes and embryos are centrifuged for a time sufficient to polarise cytoplasmic lipid from the oocytes and embryonic cells to the outside of the cells, for example 1 to 15 minutes at 10,000 to 20,000g. The time period of centrifugation will depend upon the centrifugal force applied during centrifugation. At a centrifugal force of about 13000g polarisation takes place after about 8 minutes of centrifugation. It may be more convenient to centrifuge the oocytes and embryos in the presence of embryo culture medium, rather than in the presence of more viscous cryoprotective-containing solutions.

Culture medium and cryoprotectant-containing solution for culture either before or after centrifugation may contain inhibitors of actin polymerisation such as Cytochalasin B which relaxes cytoskeletal elements.

Following centrifugation to polarise lipid, the lipid polarised oocytes and embryos are subject to low temperature conditions in the presence of a cryoprotectant which results in freezing of the oocyte or embryo prior to lipid depolarisation. By lipid depolarisation is meant the return of lipid to the cells which lipid was polarised outside the cells by centrifugation. It is to be noted that on polarisation cytoplasmic lipid may be attached to cells but displaced outside the cells.

Low temperature conditions may be provided by slow cooling, rapid freezing and vitrification. In these techniques the oocyte or embryo is frozen before the lipid returns to the cells. Vitrification may take place by placing the oocytes or embryos in a vessel, and plunging the vessel into an extremely cold environment, such as liquid nitrogen or other liquefied and/or gaseous extremely cold substance. Alternatively, a vessel containing oocytes or embryos may be rapidly frozen in an ultra-cold freezer, for example at temperatures below about -30°C. Any other apparatus or methods that enable rapid freezing may be used. In one example, oocytes or embryos may be loaded into a straw which is heat sealed, and then plunged into liquid nitrogen. In another example, embryos may be pulled by capillary action into a open pulled straw, which is then plunged into liquid nitrogen and subsequently stored (Vajta et al (1997) Cryoletters 18 191-5).

Oocytes and embryos may be stored in a conventional freezer facility, at temperatures, for example, from -10°C to -70°C or more.

Frozen lipid polarised oocytes or embryos may be thawed according to conventional oocyte and embryo thawing techniques, such as incubation of a frozen straw at a temperature of 35°C to 39°C in a suitable culture medium. Thawed embryos may be washed in culture medium, further cultured briefly, and then transferred to a synchronised recipient female, such as to the uterus of a pregnancy competent female animal. At the conclusion of

pregnancy term, that is embryo development to term, the introduced embryos have developed to live animals.

The present invention provides a simple and straight forward procedure for cryopreservation of oocytes and embryos, particularly zona intact frozen embryos. On thawing and implantation of the embryos into the uterus of a pregnancy competent animal, animals may be produced in a manner which has not been achievable by the prior art.

In accordance with another aspect there is provided an animal when produced from an oocyte (subsequently fertilised) or embryo which has been cryopreserved in accordance with the invention hereinbefore described.

Oocytes or embryos may be subject to genetic manipulation prior to the process of this invention. In this regard one or more genes of interest may be inserted into an oocyte or embryo by established techniques, such as using pronuclear microinjection, homologous recombination using embryonic stem cell technologies and other established techniques for introducing genes into oocytes and embryos (Nottle et al (1997), Reprod Fertil Suppl **52**, 237-244.

This invention allows banks or libraries of embryos or oocytes to be prepared. These banks or libraries may be provided in frozen form and presented in a convenient manner. Examples include a straw or tube, and a plurality of straws or tubes with multiple oocytes or embryos. The bank or library may contain oocytes or embryos from different animals and may find use in artificial insemination and breeding programs.

Certain embodiments of the present invention will now be exemplified with reference to the following non-limiting examples.

Example 1

Porcine embryos were collected and washed thoroughly in culture medium (mPB1 - Quinn et al (1982) J. Reprod. Fert. 66:161-168) of 39°C three times.

The embryos, in the early blastocyst stage were cultured for 40 minutes in the standard embryo culture medium NCSU-23 (Peters & Reed (1991), Theriogenology 35, 253) with 10% foetal bovine serum (FBS) containing 7.5µg/ml Cytochalasin B at 39°C in a humidified environment of 5% CO_2 and air. After a five minute period of cooling from 39°C to 25°C, the embryos were cultured for 5 minutes in 6% BSA in BECM-3h, and then washed in 25% VS3a for approximately three minutes (VS3a containing 6.5 m glycerol, 6% bovine serum albumin in BECM-3h (Dobrinsky et al (1996) Biol. Reprod. 55: 1069-1074). The embryos were centrifuged in a 1.5 Eppendorf tube (in the same media) at 13000g for about 12 minutes, recovered back into 25% VS3a, and then left in that media for a further five minutes. The embryos were then washed for 30 seconds in 65% VS3a, followed by a wash in 1ml VS3a before being loaded into a straw, heat sealing the straw, and plunging the embryos in the straw into liquid nitrogen.

In an alternative to storage in a heat-sealed straw, the embryos following a wash in the vitrification solution, are placed into a small drop of vitrification solution and drawn by capillary action into a narrowed hand pulled 0.25ml freezing straw (unsealed pulled straw, UPS). The straw is then plunged directly into liquid nitrogen.

Results of one experiment are set forth in Table 1.

TABLE 1

Freezing Method	Experimental Replicates	Embryo Stage	Embryo Number	Number viable (%) with blastocoels after culture for 24h	48h
Standard freezing method (BEVS)	2	Mor	4	0	0
	2	MBI	17	0	0
	2	Peri	13	2(15.4)	2(15.4)
BEVS/Sp	2	Mor	11	5(45.5)	6(54.6)
	3	MBI	43	27(62.8)	26(60.5)
BEVS/UPS	3	Peri	17	10(58.8)	10(58.8)
BEVS/Sp/UPS	2	Mor	6	3(50)	5(83.3)
	5	MBI	44	33(75)	28(63.6)

TABLE 1

Survival of Porcine Embryos frozen at various stages, thawed and then cultured for 48h. The morphological stages examined were Mor, morulae, MBI, early to mid blastocysts; Peri, peri-hatching blastocysts. The treatment used were BEVS, Beltsville embryo vitrification system (Dobrinsky et al (1997) Theriogenology 47:, 343); Sp, centrifuged, UPS, unsealed pulled straw.

Example 2

Method

Embryos were collected, washed thoroughly in mPB1 and then cultured for 35 minutes in NCSU-23 + 10% FBS with 7.5µg/ml Cytochalasin B at 39°C in an atmosphere of 5% CO_2 in air and 100% humidity. Morulae to middle blastocyst stages were centrifuged at 13000g for the last 10 minutes of this incubation in the culture medium containing the Cytochalasin B.

The embryos are then transferred to 2M ethylene glycol in mPB1 at 25°C for five minutes before being washed thoroughly in 8M ethylene glycol and 7% PVP in PBS and then placed into a small droplet of the vitrification media and loaded into an unsealed pulled glass by capillary action. The straw is then plunged into liquid nitrogen and stored.

Thawing is by placing the end of the straw containing the embryos into 1.2mls of 1M sucrose in PBS at 39°C. By blocking the open end with a finger, the fluid containing the embryos is forced out once, thawed, by the warming of the straw. Once collected, the embryos are placed into 1M ethylene glycol in mPBI for two minutes followed by 0.5M ethylene glycol in mPBI for a further 2 minutes, both at 25°C. Five minutes in mPB1 at 39°C completes the rehydration procedure. The embryos can then be prepared for culture or transfer.

TABLE 2

Results

Freezing Method	Embryo Stage	Replicates	Embryo Number	Number Viable (%) with blastocoels after culture for 24hrs	48hrs
E/UPS	MB1	1	5	0(0)	0(0)
	Peri	3	18	8(44.4)	6(33.3)
E/Sp/UPS	MB1	2	14	12(85.7)	11(78.6)

TABLE 2

Survival of freshly collected porcine embryos, vitrified with 8m ethylene glycol and 7% PVP. Embryos stages were early to middle blastocysts (MBI) and peri-hatching blastocysts (Peri). Freezing methods were: E/UPS, vitrified in the medium described above in an unsealed pulled straw, and E/Sp/UPS, vitrified in a similar manner but centrifuged at 13000g during the last 10 minutes of the culture in NCSU-23 + Cytochalasin B (7.5µg/ml).

Transfer of Vitrified Embryos

The embryos once thawed using the appropriate technique are washed 3 times in mPB1 at 39°C before being held in mPB1 until just prior to transfer. They are then washed in media (PBS + 10% FBS + 2% Penicillin/Streptomycin solution (CSL: Penicillin G 5000µ/ml,

Streptomycin sulphate 5000μg/ml)) at 39°C before loading into a Tomcat Catheter attached to a 1ml syringe followed by immediate transfer into one horn of the recipient animal.

TABLE 3

Experiment Number	Day of Transfer	Embryonic Stage	Embryo Number	Vitrification Technique	Results thus far
1	4	Early blastocysts	37	BEVS/Sp/UPS	Returned
2	4	Early blastocysts	32	BEVS/Sp/UPS	Returned
3	4	Peri-hatching (16 hatched)	37	BEVS/UPS	Pregnant 5 piglets born alive
4	4	Peri-hatching	32	BEVS/UPS	Pregnant 3 piglets born alive

TABLE 3

Results of the transfer of porcine embryos, vitrified and thawed, into pseudopregnant recipients. Techniques used were: BEVS, Beltsville Embryo Vitrification System: Sp, centrifugation as described in methods: UPS, vitrified and stored in an unsealed pulled straw. This recipient gave birth to 5 normal healthy piglets. A fourth recipient (Experiment 4) was also confirmed pregnant at 35 days gestation and produced 3 normal healthy piglets.

The two recipients from the Experiment 2 were confirmed as not pregnant 42 days after ovulation by progesterone assay. This was followed by a return to oestrus. The third recipient (Experiment 3) was confirmed as pregnant by progesterone assay 35 days after ovulation and by detection of a uterine artery pulse.

TABLE 4

Recipient No.	Day of Transfer	Embryonic Stage	Embryonic Number	Results
1	4	Early blastocyst Zona intact	36	Pregnant 5 piglets born alive
2	4	Early blastocyst Zona intact	36	Delayed return
3	4	Early blastocyst Zona intact	38	Delayed return
4	4	Early blastocyst Zona intact	37	21 day return

TABLE 4

Results of the transfer of zona intact early blastocysts. The embryos were centrifuged during the pre-incubation period with Cytochalasin B, vitrified with 8 methyleneglycol and 7% PVP while stored in unsealed pulled straws.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising" or the term "includes" or variations thereof, will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers. In this regard, in construing the claim scope, an embodiment where one or more features is added to any of claims is to be regarded as within the scope of the invention given that the essential features of the invention as claimed are included in such an embodiment.

CLAIMS

1. A method for the cryopreservation of oocytes or embryos, which comprises centrifugation of oocytes or embryos to polarise cytoplasmic lipid outside the oocyte or embryonic cells, subjecting the oocytes or embryos to low temperature conditions in the presence of a cryoprotectant which results in freezing of the oocytes or embryos prior to lipid depolarisation, followed by low temperature storage of the frozen lipid polarised oocytes or embryos.

2. A method according to claim 1 wherein the embryos are zona intact embryos.

3. A method according to claim 1 wherein oocytes and embryos are obtained from companion animals (for example dogs and cats), and domestic/livestock animals (for example horses, cows, sheep, goats, llamas and alpacas).

4. A method according to claim 3 wherein the oocytes and embryos are porcine oocytes and embryos.

5. A method according to claim 1 wherein the embryos are vitrified in the presence of a solution containing one or more cryoprotectant agents.

6. A method according to claim 2 wherein the cryoprotectants are selected from dimethylsulfoxide, propylene glycol, ethylene glycol, glycerol, PVP, sucrose, trehalose, Ficoll, acetamide and egg yolk.

7. A method for producing live animals from embryos which comprises thawing a cryopreserved zona intact embryo produced according to claim 1 and thereafter introducing the embryo into the uterus or fallopian tubes of a pregnancy competent female pig which at the conclusion of pregnancy term gives rise to live piglets.

8. An animal produced form a cryopreserved oocyte or embryo produced according to claim 1.

9. An animal produced according to the method of claim 7.

10. An animal according to claim 7 which is a pig.

11. A cryopreserved oocyte or embryo when produced according to claim 1.

12. A method according to claim 3 wherein embryos are vitrified by freezing in liquid nitrogen.

13. A method according to claim 10 wherein oocytes or embryos are frozen in a freezing vessel including cryologic vials or freezing straws including open pulled straws in which the oocytes or embryos are located by capillary action.

14. A method according to claim 2 wherein the embryos are in the morulae to mid-blastocyst stage.

SOLE/JOINT

DECLARATION AND POWER OF ATTORNEY

As the below named inventors, we hereby declare that our residence, post office address and citizenships are as stated below next to our names: that we verily believe we are the original and joint inventors of the subject matter claimed and for which a patent is sought in the application entitled:

CRYOPRESERVATION OF OOCYTES AND EMBRYOS AND METHODS FOR PRODUCING ANIMALS INVOLVING THE SAME

which application is:

☐ the attached application (for original application)

☑ Application No. _____ filed May 24, 2001, and amended on May 24, 2001 (for declaration not accompanying application)

that we have reviewed and understand the contents of the specification of the above-identified application, including the claims, as amended by any amendment referred to above; that we acknowledge our duty to disclose information of which we are aware which is material to the patentability of this application under 37 C.F.R. § 1.56, that we hereby claim priority benefits under Title 35, United States Code §119, §172 or §365 of any provisional application or foreign application(s) for patent or inventor's certificate listed below and have also identified on said list any foreign application for patent or inventor's certificate on this invention having a filing date before that of any foreign application on which priority is claimed:

Application Number	Country	Filing Date	Priority Claimed
AU99/01048	PCT	November 24, 1999	YES
PP 7299	AUSTRALIA	November 24, 1998	YES

We hereby claim the benefit of Title 35, United States Code §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in a listed prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, We acknowledge our duty to disclose any information material to the patentability of this application under 37 C.F.R. 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Filing Date	Status

We hereby appoint John H. Mion, Reg. No. 18,879; Thomas J. Macpeak, Reg. No. 19,292; Robert J. Seas, Jr., Reg. No. 21,092; Darryl Mexic, Reg. No. 23,063; Robert V. Sloan, Reg. No. 22,775; Peter D. Olexy, Reg. No. 24,513; J. Frank Osha, Reg. No. 24,625; Waddell A. Biggart, Reg. No. 24,861; Louis Gubinsky, Reg. No. 24,835; Neil B. Siegel, Reg. No. 25,200; David J. Cushing, Reg. No. 28,703; John R. Inge, Reg. No. 26,916; Joseph J. Ruch, Jr., Reg. No. 26,577; Sheldon I. Landsman, Reg. No. 25,430; Richard C. Turner, Reg. No. 29,710; Howard L. Bernstein, Reg. No. 25,665; Alan J. Kasper, Reg. No. 25,426; Kenneth J. Burchfiel, Reg. No. 31,333; Gordon Kit, Reg. No. 30,764; Susan J. Mack, Reg. No. 30,951; Frank L. Bernstein, Reg. No. 31,484; Mark Boland, Reg. No. 32,197; William H. Mandir, Reg. No. 32,156; Scott M. Daniels, Reg. No. 32,562; Brian W. Hannon, Reg. No. 32,778; Abraham J. Rosner, Reg. No. 33,276; Bruce E. Kramer, Reg. No. 33,725; Paul F. Neils, Reg. No. 33,102; Brett S. Sylvester, Reg. No. 32,765; Robert M. Masters, Reg. No. 35,603 and George F. Lehnigk, Reg. No. 36,359, our attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and request that all correspondence about the application be addressed to SUGHRUE, MION, ZINN, MACPEAK & SEAS, PLLC, 2100 Pennsylvania Avenue, N.W., Washington, D.C. 20037-3213.

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date 13-8-01 First Inventor Mark (First Name) Brenton (Middle Initial) ~~NOTTLE~~ (Last Name)

Residence South Australia (City) AUSTRALIA (State/Country) Signature _____

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Citizenship JAPANESE JPX

Date Sixth Inventor
First Name Middle Initial Last Name

Residence Signature
City State/Country

Post Office Address:

Citizenship

Applicant or Patentee:	**Mark Brenton NOTTLE, Ranald CAMERON, Luke Francis Sharkerley BEEBE, Alan Weaver BLACKSHAW and Hiroshi NAGASHIMA**	Attorney's Docket No.:	Q-64691
Application or Patent No.			
Filed or Issued:	**May 24, 2001**		
For:	**CRYOPRESERVATION OF OOCYTES AND EMBRYOS AND METHODS FOR PRODUCING ANIMALS INVOLVING THE SAME**		

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27 (c)) --

NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION	THE UNIVERSITY OF QUEENSLAND
ADDRESS OF ORGANIZATION	St. Lucia, Queensland, 4067 AUSTRALIA

TYPE OF ORGANIZATION

☑ University or other institution of higher education

☐ Tax exempt under Internal Revenue Service Code (26 USC 501(a)) and 501(c) (3))

☐ Nonprofit scientific or educational under statute of state of The United States of America

(Name of State) _____

(Citation of statute) _____

☐ Would qualify as tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c) (30)) if located in

The United States of America

☐ Would qualify as nonprofit scientific or educational under statute of state of The United States of America if located in

The United States of America

(Name of State) _____

(Citation of statute) _____

I hereby declare that the above nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled: **CRYOPRESERVATION OF OOCYTES AND EMBRYOS AND METHODS FOR PRODUCING ANIMALS INVOLVING THE SAME** by inventors Mark Brenton NOTTLE, Ranald CAMERON, Luke Francis Sharkerley BEEBE, Alan Weaver BLACKSHAW and Hiroshi NAGASHIMA.

Described in ☐ the specification filed herewith

☑ application no. _____ filed May 24, 2001

☐ patent no. _____ issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statement are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME BRESAGEN LIMITED

ADDRESS 38-39 Winwood Street, Thebarton, South Australia, 5031, AUSTRALIA

☐ INDIVIDUAL ☑ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING DOUGLAS PORTER

TITLE IN ORGANIZATION SECRETARY AND REGISTRAR

ADDRESS OF PERSON SIGNING THE UNIVERSITY OF QUEENSLAND QLD 4072

Signature Douglas Porter Date 27/6/2001

Applicant or Patentee: **Mark Brenton NOTTLE, Ranald CAMERON, Luke Francis Sharkerley BEEBE, Alan Weaver BLACKSHAW and Hiroshi NAGASHIMA** Attorney's Docket No.: Q-64691

Application No.

Filed or Issued: **May 24, 2001**

For: **CRYOPRESERVATION OF OOCYTES AND EMBRYOS AND METHODS FOR PRODUCING ANIMALS INVOLVING THE SAME**

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27 (c)) -- SMALL BUSINESS CONCERN

I hereby declare that I am

☐ the owner of the small business concern identified below:
☑ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN BRESAGEN LIMITED

ADDRESS OF CONCERN 38-39 Winwood Street, Thebarton, South Australia, 5031, AUSTRALIA

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled: **CRYOPRESERVATION OF OOCYTES AND EMBRYOS AND METHODS FOR PRODUCING ANIMALS INVOLVING THE SAME** by inventors Mark Brenton NOTTLE, Ranald CAMERON, Luke Francis Sharkerley BEEBE, Alan Weaver BLACKSHAW and Hiroshi NAGASHIMA

Described in ☐ the specification filed herewith
☑ application no. _____ filed May 24, 2001
☐ patent no. _____ issued _____

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statement are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME THE UNIVERSITY OF QUEENSLAND

ADDRESS St. Lucia, Queensland, 4067, AUSTRALIA

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☑ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: MR LINTON WAYNE PAUL BURNS

TITLE IN ORGANIZATION: COMPANY SECRETARY

ADDRESS OF PERSON SIGNING: 38-39 WINWOOD ST THEBARTON SA 5031

Signature: _____ Date: 8 JUNE 2001